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14. ABSTRACT Preclinical work has demonstrated that glibenclamide administration improves outcomes in rat models of spinal cord injury, with the principal mechanism of action being amelioration of post-traumatic hemorrhagic necrosis (PHN). We hypothesize that some but not all patients with spinal cord injury, principally those with incomplete lesions, will respond to glibenclamide therapy. Our goal is to determine whether MRI and circulating biomarkers can be used as early markers of injury that can be used to predict which patients may benefit from glibenclamide treatment. During the first year of this grant acute MRI images (6 and 24 hours post-injury) were collected from (n=36) rats subject to spinal contusion injury. Both the severity and location of injury were changed to create six different experimental groups: (1) Midline (M) severe (50 mm); (2) M moderate (25 mm); (3) M light (12.5 mm); (4) Lateral (L) severe (50 mm); (5) L moderate (25 mm) and (6) L light (12.5 mm). Preliminary analyses of these data reveal that injury location and severity affect the rate and magnitude of secondary hemorrhage. In year 2, we compared the efficacy of glibenclamide in the two most consistent injury models (i.e., groups 2 vs. 5; moderate midline vs. lateral SCI). Data from these studies were recently published and indicate that glibenclamide is beneficial in both models of cervical SCI; however, the magnitude of benefit was greatest when the magnitude and extent of primary hemorrhage was limited during the first 24h (i.e., moderate lateral SCI).					
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INTRODUCTION:

The magnitude of acute post-traumatic hemorrhagic necrosis (PHN) is an early prognostic indicator of long-term functional recovery in human spinal cord injury (SCI). Recent preclinical data indicate that PHN can be reduced and functional recovery improved in spinal injured rats using glibenclamide (Glib), an FDA approved anti-diabetic drug that targets SUR1 receptors on endothelia. We recently generated new data suggesting that only a subset of spinal cord injuries will respond to Glib. This may also be true for other therapies that are designed to attenuate PHN. We predict that this is because of spinal cord anisotropy and differences in gray and white matter elasticity. Accordingly, studies in Aim 1 used a rat model of spinal contusion injury to determine whether varying the location and severity of trauma influences the magnitude of primary hemorrhage and the spread of secondary hemorrhage, i.e., PHN. Acute T2 MRI data show that injury location and severity of injury affect the rate and magnitude of PHN. In year 2, a recent publication documents the efficacy of glibenclamide in the context of varying the parameters of primary SCI. Namely, although glibenclamide was efficacious in all SCI rats, the most striking anatomical and functional improvements were observed when primary hemorrhage/edema was localized to the spinal hemi-cord during the first 24 hours. Ongoing experiments continue to explore the utility of using MRI as an acute biomarker for predicting glibenclamide efficacy. Also, optimal protocols are being designed to determine whether serum biomarkers will be useful as a cross-referencing strategy with acute MRI to predict glibenclamide efficacy and outcome after SCI. We predict that only a subset of animals will respond to Glib therapy and that the range of injuries created in our animal models will be analogous to at least two major subclasses of human SCI: (1) those that are neurologically complete within 24 hours post-injury and that are largely untreatable by available methods and (2) those that are incomplete but are at risk to slowly evolve into complete lesions as a result of PHN.

BODY:

The data reported below were completed at The Ohio State University and The University of Maryland and correspond with the approved statement of work. Relevant Aims, Tasks and milestones are highlighted.

Aim 1: Compare patterns of acute post-hemorrhagic necrosis, delayed secondary neurodegeneration and chronic functional recovery after varying the location and magnitude of a spinal contusion injury.

Milestone 1: Create optimized T2 MRI protocols that enable an accurate measurement of acute intraspinal hemorrhage in the different models of SCI.

- We have successfully used MRI to visualize gross lesion morphology and, with University of Maryland, we have applied T2-weighted MRI techniques to confirm that glibenclamide reduces acute intraspinal hemorrhage and/or post-traumatic hemorrhagic necrosis (see below). However, at Ohio State, we

continue to perfect the MRI methodology. Even though T2 MRI images have been acquired, we are experiencing difficulties in distinguishing the pathologic significance and boundaries of MRI hypo- and hyper-intensities. Early after injury, these radiologic signatures correlate beautifully with bleeding and edema as predicted (also see Fig. 1 in Simard et al.; appendix). However, at later times post-injury, these correlations are not absolute and subtle differences between injury types make boundary distinctions difficult. Histologic correlations and ex vivo MRI analyses are needed to help inform the true nature of the hyper/hypointensities present in vivo MRI images. Histologic analyses are ongoing and a no-cost extension of existing funds is needed to complete additional MRI studies.

Milestone 4: Document injury-dependent efficacy of glibenclamide based on T2 MRI

- Completed. As shown in the attached manuscript (Simard et al., 2013), glibenclamide has a clear effect on reducing intraspinal hemorrhage. Specifically, glibenclamide decreases hemorrhage, as measured in vivo from MRI T2 hypointensities, ~50% by 24h post-injury. These data indicate that acute MRI analyses could have prognostic value in evaluating drug efficacy (e.g., comparing MRI signatures within 24h across treated/non-treated subjects). As described above, ongoing studies are using MRI to determine if changes in injury location differentially affect intraparenchymal hemorrhage at early and chronic post-injury time periods and whether specific types of SCI may be more amenable to glibenclamide therapy than other types (Milestone 4). Future attempts to intervene using glibenclamide will be based on robustness of data from these ongoing experiments.

KEY RESEARCH ACCOMPLISHMENTS:

- We published data indicating that MRI can be used to detect dynamic changes in lesion size following glibenclamide therapy.

REPORTABLE OUTCOMES:

Simard, Popovich et al., Spinal Cord, Sep 17. Doi: 10.1038/sc.2013.99

CONCLUSION:

Acute bolus infusion of glibenclamide reduces intraspinal hemorrhage by ~50% within 24h of spinal cord injury. T2-weighted MRI has early prognostic value in predicting neuroprotection following glibenclamide therapy.

REFERENCES:

Simard, Popovich et al., Spinal Cord, Sep 17. Doi: 10.1038/sc.2013.99

APPENDICES:

Simard, Popovich et al., Spinal Cord, Sep 17. Doi: 10.1038/sc.2013.99

SUPPORTING DATA: See attached manuscript

ORIGINAL ARTICLE

MRI evidence that glibenclamide reduces acute lesion expansion in a rat model of spinal cord injury

JM Simard^{1,2,3}, PG Popovich⁴, O Tsybalyuk¹, J Caridi¹, RP Gullapalli⁵, MJ Kilbourne⁶ and V Gerzanich¹**Study design:** Experimental, controlled, animal study.**Objectives:** To use non-invasive magnetic resonance imaging (MRI) to corroborate invasive studies showing progressive expansion of a hemorrhagic lesion during the early hours after spinal cord trauma and to assess the effect of glibenclamide, which blocks Sur1-Trpm4 channels implicated in post-traumatic capillary fragmentation, on lesion expansion.**Setting:** Baltimore.**Methods:** Adult female Long–Evans rats underwent unilateral impact trauma to the spinal cord at C7, which produced ipsilateral but not contralateral primary hemorrhage. In series 1 (six control rats and six administered glibenclamide), hemorrhagic lesion expansion was characterized using MRI at 1 and 24 h after trauma. In series 2, hemorrhagic lesion size was characterized on coronal tissue sections at 15 min (eight rats) and at 24 h after trauma (eight control rats and eight administered glibenclamide).**Results:** MRI (T2 hypodensity) showed that lesions expanded 2.3 ± 0.33 -fold ($P < 0.001$) during the first 24 h in control rats, but only 1.2 ± 0.07 -fold ($P > 0.05$) in glibenclamide-treated rats. Measuring the areas of hemorrhagic contusion on tissue sections at the epicenter showed that lesions expanded 2.2 ± 0.12 -fold ($P < 0.001$) during the first 24 h in control rats, but only 1.1 ± 0.05 -fold ($P > 0.05$) in glibenclamide-treated rats. Glibenclamide treatment was associated with significantly better neurological function (unilateral BBB scores) at 24 h in both the ipsilateral (median scores, 9 vs 0; $P < 0.001$) and contralateral (median scores, 12 vs 2; $P < 0.001$) hindlimbs.**Conclusion:** MRI is an accurate non-invasive imaging biomarker of lesion expansion and is a sensitive measure of the ability of glibenclamide to reduce lesion expansion.

Spinal Cord advance online publication, 17 September 2013; doi:10.1038/sc.2013.99

Keywords: spinal cord injury; glibenclamide; riluzole; Sur1-Trpm4 channel; MRI; progressive hemorrhagic necrosis

INTRODUCTION

Spinal cord injury (SCI) remains one of the foremost unsolved challenges in medicine. Worldwide, the incidence of SCI ranges from 10 to 83 per million people per year, with half of these patients suffering a complete lesion and one-third becoming tetraplegic.¹ At present, little can be done to undo or repair the initial damage to spinal cord tissues, but great hope lies in reducing secondary injury processes triggered by the primary injury that increase the damage and worsen clinical outcome.

Histological studies on animal models of SCI have shown that early expansion of a hemorrhagic contusion is a common feature following trauma to the spinal cord. During the hours after a blunt impact, a dynamic process ensues wherein a hemorrhagic contusion slowly enlarges, resulting in the progressive autodestruction of spinal cord tissues.^{2–4} Discrete petechial hemorrhages appear, first around the site of injury and then in more distant areas.⁵ As petechial hemorrhages form and coalesce, the lesion gradually expands, with a characteristic region of hemorrhage that ‘caps’ the advancing front of the lesion. A small hemorrhagic lesion that initially involves primarily the capillary-rich gray matter doubles in size during the first 24 h after

injury. The advancing hemorrhage results from delayed progressive catastrophic failure of the structural integrity of capillaries, a phenomenon termed ‘progressive hemorrhagic necrosis’.^{2–4}

Progressive hemorrhagic necrosis has been linked to *de novo* upregulation of sulfonylurea receptor 1 (Sur1)—transient receptor potential melastatin 4 (Trpm4) channels (a.k.a., Sur1-regulated NC_{Ca}-ATP channels)⁶ in microvessels.^{4,7,8} Sur1-Trpm4 channels have been shown to be responsible for the necrotic death of endothelial cells that results in delayed fragmentation of capillaries and formation of petechial hemorrhages. Gene suppression as well as pharmacological blockade of Sur1-Trpm4 channels prevents progressive hemorrhagic necrosis, reduces lesion size and significantly improves neurological function in rodent models of SCI.^{7–10}

To date, the magnitude and time course of lesion expansion due to progressive hemorrhagic necrosis have been characterized in rat models by measuring the amount of extravasated blood in the spinal cords of animals that were killed at different times after trauma.⁴ Although an important measure of injury, quantifying the amount of extravasated blood may not accurately reflect the actual volume of compromised tissue, as this technique cannot distinguish between an

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increase in the number of erythrocytes within a given volume of tissue versus an actual increase in volume of contused tissue affected by hemorrhage and edema. We hypothesized that magnetic resonance imaging (MRI) could be used to assess early lesion expansion non-invasively and to independently corroborate the existence of early lesion expansion due to progressive hemorrhagic necrosis after spinal cord trauma. Here, we used serial MRI scans obtained at 1 and 24 h after trauma to characterize lesion expansion, and we validated our MRI measurements by comparing them with measurements based on coronal tissue sections in a different group of rats with the same injury. In these experiments, we also examined the effect of glibenclamide on lesion expansion and on neurological function.

MATERIALS AND METHODS

Ethics statement

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, Baltimore, and in accordance with the relevant guidelines and regulations as stipulated in the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. In accordance with 'good laboratory practice', different investigators blinded to injury-group conducted behavioral tests and analyzed the data.

Subjects and experimental series

These experiments were conducted on new series of animals distinct from those previously reported from this laboratory. Thirty-six female Long-Evans rats (250–300 gm; Harlan, Indianapolis, IN, USA) were used in two experimental series (Table 1). In series 1, 12 rats underwent an SCI (see below), six were administered glibenclamide (see below) and six served as untreated controls; these 12 rats were studied using MRI at 1 and 24 h after trauma, after which they were euthanized. In series 2, 24 rats underwent an SCI; eight were euthanized at 15 min; of the remaining 16 rats, eight served as untreated controls and eight were administered glibenclamide; these 16 rats were assessed for locomotor function at 24 h, after which they were euthanized to obtain tissue sections for measurements of the hemorrhagic lesion area at the epicenter.

Sample size calculation

Previous experiments with the model used here suggested that an effect size (Cohen's *d*) of 2.27 (means, 2 vs 1 μ l; pooled s.d., 0.44 μ l) might be expected

for hemorrhage volumes with vehicle vs glibenclamide treatments.⁴ Sample size calculation for a 2-sample comparison ($\alpha = 0.05$; 2-tailed), an effect size of 2.27, and a desired power of 90% indicated a minimum sample size of six per group.

Rat model of SCI

A unilateral impact to the cervical spinal cord at C7 was calibrated to produce ipsilateral but not contralateral primary hemorrhage, resulting in severe SCI, as described in detail previously.^{4,8–10} Female Long-Evans rats (~11 weeks; 250–300 gm; Harlan) were anesthetized (Ketamine, 60 mg kg⁻¹ plus Xylazine, 7.5 mg kg⁻¹, intraperitoneally (IP)). On the left side, the entire lamina of C7 and the dorsal half of the pedicle of C7 were removed. The dura was exposed by removing the interlaminar ligaments rostral and caudal to the lamina of C7 and any remaining ligamentum flavum. The guide tube containing the impactor (1.55-mm tip diameter, 57 mm length, 1.01 gm)¹¹ was angled 5° medially and was positioned using the manipulator arm of the stereotaxic apparatus. The impactor was activated when struck by a 10-gm weight dropped from a height of 25 mm inside the guide tube (velocity, 0.7 m s⁻¹). After injury, rats were nursed on a heating pad to maintain a rectal temperature of ~37 °C and were given 10 ml of glucose-free normal saline subcutaneously. Food and water were placed within easy reach to ensure that the rats were able to eat and drink without assistance. Bladder function was assessed 2–3 times daily by observing for urination, and, if needed, manual emptying of the bladder was carried out using the Credé maneuver.

Treatment

Treatment consisted of the following: (1) administering a single loading dose of glibenclamide (10 μ g kg⁻¹) or an equivalent volume of vehicle IP, at 5 min after trauma; and (2) implanting a mini-osmotic pump (Alzet 2001, 1.0 μ l h⁻¹; Alzet Corp., Cupertino, CA, USA) for continuous subcutaneous infusion of glibenclamide (200 μ g ml⁻¹) beginning at the end of surgery, resulting in delivery of 200 ng h⁻¹ or an equivalent volume of vehicle subcutaneously until euthanasia. This dosing regimen was found previously to significantly ameliorate progressive hemorrhagic necrosis^{8–10} without producing clinically relevant hypoglycemia or any other toxicity.^{4,12,13} Unlike our previous study, in which a 3-h treatment delay was used,⁹ here we used an early treatment time because our principal goal was to validate MRI for measuring early lesion expansion. The formulation of glibenclamide (#G2539; Sigma, St Louis, MO, USA) in dimethyl sulfoxide (DMSO) and the preparation of mini-osmotic pumps have been described in detail.¹⁴ Briefly, a stock solution was prepared by placing 25 mg of glibenclamide into 10 ml of DMSO. The solution used for the loading dose was prepared by adding 4 μ l of stock solution to 1 ml unbuffered normal saline. The solution for infusion was prepared by taking 2.3-ml unbuffered normal saline, adding 4 μ l of 10 N NaOH (undiluted Fixanal; Riedel-deHaën, Seelze, Germany), and then adding 200- μ l stock solution, in that order in order to prevent precipitation of the drug. Control animals received vehicle solution prepared as above but without glibenclamide.

Measurements of primary and secondary hemorrhage

In series 2, at the designated time after injury (15 min for primary hemorrhage; 24 h for primary plus secondary hemorrhage), the rat was anesthetized with sodium pentobarbital (100 mg kg⁻¹), a thoracotomy was performed and the rat was transcardially perfused with normal saline (50 ml) at a pressure of 100 cm H₂O. The spinal cord was collected and sectioned coronally through the epicenter of injury, and the face of the epicenter was imaged at high resolution (1200 dpi) using a flat-bed scanner (Epson Perfection 2450 Photo).

Locomotor function

Basso, Beattie and Bresnahan (BBB) scores were determined as described,¹⁵ except that modifications were introduced to allow more accurate assessment of the functional asymmetry associated with hemicord injury. We refer to this modified functional assessment as modified (unilateral) BBB scores.^{9,10}

Table 1 Experimental series

Treatment		Intervention		
		15 min	1 h	24 h
Series 1				
Group 1 (n=6)	Control		MRI	MRI euthanize
Group 2 (n=6)	Glibenclamide		MRI	MRI euthanize
Series 2				
Group 1 (n=8)	Control	Euthanize section spinal cord		
Group 2 (n=8)	Control			mBBB score euthanize section spinal cord
Group 3 (n=8)	Glibenclamide			mBBB score euthanize section spinal cord

Abbreviations: mBBB, modified (unilateral) Basso, Beattie and Bresnahan; n, number of rats

Magnetic resonance imaging

In series 1, all rats were imaged 1 and 24 h after trauma; four control rats also were imaged 6 h after trauma. The delay of 1 h in obtaining the 'baseline' MRI was necessitated by logistical considerations, principally the distance between the site where injuries were produced and the site where MRIs were obtained. MRI was performed on a 3.0 Tesla Siemens Trio MR scanner equipped with 18 receiver channels and high performance gradients ($200 \text{ mTm}^{-1} \text{ ms}^{-1}$). An eight-channel wrist coil was used to image the rats. Anesthetized (Ketamine, 60 mg kg^{-1} plus Xylazine, 7.5 mg kg^{-1} , IP) animals were placed in the supine position with the 'sweet spot' of the coil centered on the cervical spine of the rat. The depth of anesthesia was monitored continuously during the imaging session.

The imaging sequences consisted of localizers in the three orthogonal planes. Following localization, T1-weighted magnetization prepared rapid acquisition gradient echo (MP-RAGE) images were acquired in the axial plane with adequate coverage of the injury using 64 slices over a field of view of $53 \text{ mm} \times 62 \text{ mm}$ and an interpolated pixel resolution of 448×512 . The imaging parameters were as follows: inversion time $\text{TI} = 900 \text{ ms}$ and $\text{TE/TR} = 4 \text{ ms}/1900 \text{ ms}$ for an acquisition time of 3 min and 53 s using an acceleration factor of 2. T2-weighted 3D spin-echo images were then acquired in the sagittal and the axial plane. For both planes, the imaging parameters were as follows: $\text{TE/TR} = 93 \text{ ms}/1000 \text{ ms}$, 64 slices, slice thickness 0.3 mm over a field of view of $45 \text{ mm} \times 90 \text{ mm}$ at an interpolated pixel resolution of 312×640 for a total acquisition time of 5 min and 24 s using parallel imaging with an acceleration factor of 2.

All images were reconstructed in three planes to visualize the extent of the lesion. The hemorrhagic portion of the contusion was identified as a hypointense region (due to the presence of hemoglobin) within the cord on T2-weighted images. These regions were demarcated on each slice, and the total volume of the lesion was determined.

Data analysis

T2-hypodensity volumes at 1 versus 24 h were analyzed using Student's paired *t*-test. Areas of hemorrhagic contusion on tissue sections at the epicenter in three groups of rats (15 min untreated, 24-h vehicle, 24-h glibenclamide) were analyzed using AVOVA, with Bonferroni *post hoc* comparisons. Modified (unilateral) BBB scores in two groups of rats (vehicle vs glibenclamide) were analyzed using the Mann-Whitney *U*-test.

RESULTS

In series 1, we evaluated the expansion of the hemorrhagic contusion using serial MRI scans obtained at 1 and 24 h after trauma, with the T2 hypodensity being taken as a measure of extravasated blood.^{16–19} In control rats, a small T2 hypodensity was evident at the first examination, obtained 1 h after trauma (Figure 1a). Subsequent imaging obtained at 6 h ($n = 4$) and 24 h ($n = 6$) confirmed progressive enlargement of the hemorrhagic lesion (Figure 1a). In six control rats, the volume (mean \pm s.e.) of the T2 hypodensity at 1 h after trauma was $1.52 \pm 0.37 \mu\text{l}$, and, after 24 h, the volume increased 2.3-fold to $3.55 \pm 0.87 \mu\text{l}$ ($P < 0.01$) (Figure 1b). By contrast, in six rats treated with glibenclamide 5 min after trauma, the volume of the T2 hypodensity at 1 h was $0.51 \pm 0.21 \mu\text{l}$, and, after 24 h, the volume increased 1.2-fold to $0.59 \pm 0.24 \mu\text{l}$ ($P = 0.43$) (Figure 1b).

In series 2, we evaluated the size of the hemorrhagic contusion using images of tissue sections taken through the epicenter of injury at 15 min and at 24 h. We considered the hemorrhagic area observed at 15 min to represent the primary hemorrhage due to the trauma and the hemorrhagic area observed at 24 h to represent the primary hemorrhage plus the secondary hemorrhage, the latter attributable to progressive hemorrhagic necrosis.

At 15 min, a contusion was apparent that was confined largely to the ipsilateral hemicord, mostly the gray matter (Figure 2a, upper). In control rats, the hemorrhagic contusion at 24 h invariably involved a larger area, typically extending to the contralateral side (Figure 2a,

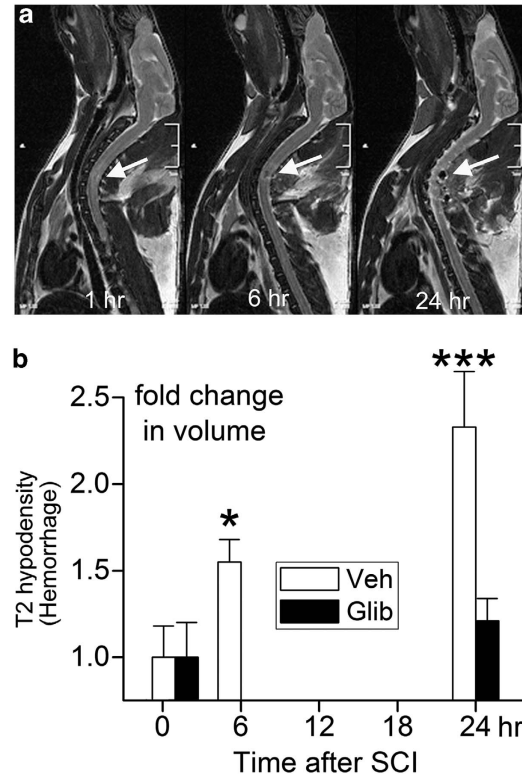


Figure 1 Progressive expansion of hemorrhagic contusion assessed using MRI. (a) Serial MRIs of a control rat obtained at 1, 6 and 24 h after trauma, showing progressive enlargement of the T2 hyperdensity due to the presence of increasing amount of hemoglobin; the data shown are representative of six rats. (b) Fold-change in volumes of T2 hyperdensity measured at the times indicated in control rats (Veh; empty bars) versus glibenclamide-treated rats (Glib; filled bars); mean \pm s.e.; six rats per group; * $P < 0.05$; *** $P < 0.001$.

middle). By contrast, in rats administered glibenclamide 5 min after trauma, the hemorrhagic contusion at 24 h typically occupied an area comparable to that observed at 15 min (Figure 2a, lower). Quantification of the hemorrhagic area showed that, in control rats, lesions were 2.2 ± 0.12 -fold larger at 24 h compared with that at 15 min. By contrast, in glibenclamide-treated rats, lesions were 1.1 ± 0.05 -fold larger at 24 h compared with that at 15 min (Figure 2b).

Commensurate with the favorable effect of glibenclamide on hemorrhagic lesion area, assessment of modified (unilateral) BBB scores at 24 h showed that glibenclamide was associated with significantly better neurological function. Median scores were 0 vs 9 ($P < 0.001$) for the ipsilateral hindlimb and 2 vs 12 ($P < 0.001$) for the contralateral hindlimb, for the control vs glibenclamide groups, respectively (Figure 2c).

DISCUSSION

The principal finding of the present study is that, based on measurements of MRI T2-lesion volume and measurements of hemorrhagic lesion area, there is a 2- to 2.5-fold increase in the hemorrhagic contusion that takes place during the first 24 h after blunt impact trauma to the spinal cord, in good agreement with previous measurements based on tissue hemoglobin.⁴ Previous histological and MRI studies in rats have characterized spinal cord lesions at various times after injury, but relatively few have examined temporal and spatial characteristics of lesion progression during the first hours after injury. To our knowledge, the earliest study

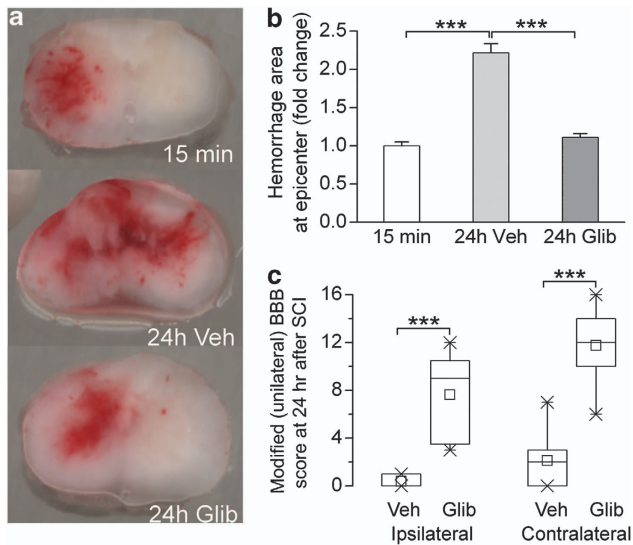


Figure 2 Progressive expansion of hemorrhagic contusion assessed from tissue sections at the epicenter. (a) Representative coronal tissue sections of perfusion-cleared but otherwise unprocessed spinal cords through the epicenter of injury 15 min (top) and 24 h (middle and bottom) after impact, in control rats (top and middle), and in a glibenclamide-treated rat (bottom); the data shown are representative of eight rats per group. (b): Fold-change in areas of hemorrhage at the epicenter in the three groups of rats depicted in (a); mean \pm s.e.; eight rats per group; *** $P < 0.001$. (c) Box plots showing modified (unilateral) BBB scores for the ipsilateral and contralateral hindlimbs at 24 h, for the two groups of rats depicted in (b), 24 h Veh and 24 h Glib; box, 25th and 75th percentiles; \times , 1st and 99th percentiles; line, median; small square, mean.

addressing this question (weight drop; midline, lower thoracic/upper lumbar) reported that, on H&E-stained sections, intramedullary hemorrhages involved an aggregate of 11% of the spinal cord area at the level of maximal bleeding immediately after trauma and that this increased 2.5-fold to 28% after 8 h.²⁰ In our previous study (weight drop; lateral C7), we reported a twofold increase in the amount of extravasated blood in tissues from the epicenter during the first 12 h after trauma.⁴ In an MRI study (0.5 mm compression for 30 msec; T7), the T2-lesion volume was found to expand ~ 1.5 -fold over 5.5 h.¹⁶ Together with the present study, these various animal studies using different methods establish conclusively the existence of significant lesion expansion during the early hours after blunt impact to the spinal cord. Recently, lesion expansion also was demonstrated using serial MRI scans in humans with cervical SCI.²¹ The importance of these observations lies in the hope that, if early lesion expansion can be halted, patients with acute SCI may suffer the least possible secondary injury.

The model of cervical hemicord impact that we used here is particularly well suited to examining the expansion of the primary injury to the contralateral side. In this model, care is taken to obtain a primary hemorrhage exclusively ipsilateral to the site of impact. This model emphasizes the distinction between primary and secondary hemorrhage, and it shows the influence of secondary hemorrhage on outcome.⁸ When primary hemorrhage is located exclusively unilaterally, subsequent spread of the hemorrhagic lesion to the contralateral side during the ensuing hours—by definition, secondary hemorrhage—is readily discerned both histologically and functionally. Moreover, if the primary hemorrhage is confined to one side and if secondary expansion of the hemorrhagic lesion to the contralateral side is prevented, the magnitude and importance of secondary

hemorrhage is readily appreciated. By contrast, with bilateral primary hemorrhage, neurological dysfunction due to secondary hemorrhage is more difficult to detect. When the primary hemorrhage already occupies most of a spinal segment, expansion laterally of secondary hemorrhage may be limited or functionally mute. Rostro-caudal expansion would still occur but, depending on the spinal cord level involved, for example, cervical vs thoracic, this may be difficult to detect neurologically.

An important aspect of the present study is that it confirmed previous observations that early lesion expansion can be prevented by blocking either of the two subunits of the Sur1-Trpm4 channel.⁶ Lesion expansion is prevented by blocking Sur1, either pharmacologically with glibenclamide or repaglinide,⁴ or by gene suppression with antisense oligodeoxynucleotide or gene deletion of *Abcc8*,⁸ or by blocking Trpm4, either pharmacologically with flufenamic acid⁷ or riluzole,⁹ or by gene suppression with antisense oligodeoxynucleotide or gene deletion of *Trpm4*.⁷

In the present study examining acute outcomes at 24 h, in three other series from our laboratory examining outcomes at 1 or 6 weeks,^{4,8,10} and in one series from an independent laboratory,²² glibenclamide treatment beginning shortly after trauma was found to be highly effective in reducing lesion size and improving neurological function. In a 6th series of rats with outcomes evaluated at 6 weeks, treatment at the clinically more relevant time of 3 h after trauma also was found to be highly beneficial.⁹ As might be expected, the magnitude of the benefit observed with glibenclamide depends on the magnitude of the primary injury,¹⁰ but all studies to date examining functional outcome and lesion size at 6 weeks have demonstrated a significant treatment effect, regardless of the initial severity.^{8–10}

In the 6 series to date with glibenclamide, drug was delivered by constant subcutaneous infusion. From a pharmacokinetic perspective, cutaneous delivery of glibenclamide is highly effective for maintaining steady plasma levels, is superior to enteral administration and appears to be equivalent to intravenous (IV) administration.²³ Constant subcutaneous infusion of glibenclamide was used in the preclinical studies as a convenient alternative to constant IV infusion, as is used with injectable glibenclamide (RP-1127) in clinical trials for other CNS indications (ClinicalTrials.gov identifiers: NCT01454154; NCT01268683; NCT01794182). In the animal studies, no clinically relevant hypoglycemia or other toxicity has been detected with infusions of 200 ng h^{-1} ,^{4,12,13} or 400 ng h^{-1} .²⁴ In a Phase I trial of RP-1127 in 16 healthy volunteers (ClinicalTrials.gov identifier: NCT01132703), a 3-day IV infusion ($125 \mu\text{g/h}$) produced no clinically significant hypoglycemia or other serious adverse event (S. Jacobson, personal communication).

A recent comprehensive review²⁵ shows how difficult it can be to translate preclinical trials on acute pharmacotherapeutic neuroprotection to clinical trials in SCI. Among numerous challenges, consideration must be given to the accumulated preclinical data on any proposed therapeutic agent as well as the effect of that agent on surrogate end points and functional outcome. The work reported here shows that early MRI may be a useful imaging biomarker of lesion expansion, a phenomenon that is prominent in human SCI,²¹ and that may serve as a useful proxy for treatment efficacy.

Riluzole has been found to be efficacious in preclinical models of SCI,^{9,26,27} may have a beneficial effect on motor outcome in cervical SCI, as recently reported in a small open-label Phase I clinical trial,²⁸ and is planned for study in a Phase II clinical trial of acute SCI (ClinicalTrials.gov identifier, NCT01597518). A recent preclinical study using a rat model of SCI, which was so severe as to have

attendant mortality, compared treatment with riluzole (2.5 mg kg⁻¹ IP every 12 h × 1 week) vs glibenclamide (200 ng h⁻¹ continuous subcutaneous infusion – 1 week), starting 3 h after trauma.⁹ This study found that glibenclamide is superior to riluzole in terms of both toxicity and efficacy. Riluzole exhibits a peculiar, dose-limiting CNS toxicity in the context of CNS trauma that is absent in non-injured controls: in SCI, mortality rates of 0, 8 and 70% were observed with 4, 6 and 8 mg kg⁻¹ IP every 12 h, respectively.²⁷ Riluzole is a pleiotropic drug with inhibitory effects on sodium channels, glutamatergic pathways and Trpm4.⁹ However, the near-identity of the phenotypes observed with riluzole⁹ compared with gene suppression of Sur1⁸ as well as gene suppression of Trpm4⁷ suggests that a major effect of riluzole in SCI is via inhibition of the Sur1-Trpm4 channel. Glibenclamide appears to be a safer choice than riluzole for targeting the Sur1-Trpm4 channel in acute CNS injury.

The present study has important shortcomings. First, it was not designed as an efficacy study, but rather to demonstrate the concept that MRI could be used to measure progressive lesion expansion in a rat model of SCI. Thus, the number of subjects studied was small, although the specific number was determined by a power analysis. The most important shortcoming was that the first MRI was obtained 1 h after trauma, whereas treatment with glibenclamide was administered 5 min after trauma. The long delay in obtaining the MRI was necessitated by logistical considerations, principally the distance between the site where injuries were produced and the site where MRIs were obtained. We believe that this problem with study design is the reason for the apparently smaller ‘baseline’ lesions in glibenclamide versus control groups, even though both groups were injured in the same way. Further experiments will be needed to examine MRIs at earlier times. However, if our interpretation is correct, it suggests that ‘hyper-early’ treatment with glibenclamide may be highly desirable for maximally improving outcome.

In conclusion, the present study reaffirms that early enlargement of the hemorrhagic contusion is an important factor determining outcome in SCI, and that reducing early enlargement of the hemorrhagic contusion with glibenclamide can contribute significantly to improved functional outcome.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

JMS holds a US patent (7,872,048), ‘Methods for treating spinal cord injury with a compound that inhibits a NC(Ca-ATP) channel’. JMS is a member of the scientific advisory board and holds shares in Remedy Pharmaceuticals. No support, direct or indirect, was provided to JMS, or for this project, by Remedy Pharmaceuticals. All other authors declare no conflict of interest.

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- Steward O, Schauwecker PE, Guth L, Zhang Z, Fujiki M, Inman D *et al*. Genetic approaches to neurotrauma research: opportunities and potential pitfalls of murine models. *Exp Neurol* 1999; **157**: 19–42.
- Guth L, Zhang Z, Steward O. The unique histopathological responses of the injured spinal cord. Implications for neuroprotective therapy. *Ann N Y Acad Sci* 1999; **890**: 366–384.
- Simard JM, Tsybalyuk O, Ivanov A, Ivanova S, Bhatta S, Geng Z *et al*. Endothelial sulfonyleurea receptor 1-regulated NC(Ca-ATP) channels mediate progressive hemorrhagic necrosis following spinal cord injury. *J Clin Invest* 2007; **117**: 2105–2113.
- Kawata K, Morimoto T, Ohashi T, Tsujimoto S, Hoshida T, Tsunoda S *et al*. Experimental study of acute spinal cord injury: a histopathological study. *No Shinkei Geka* 1993; **21**: 45–51.
- Woo SK, Kwon MS, Ivanov A, Gerzanich V, Simard JM. The sulfonyleurea receptor 1 (Sur1)-transient receptor potential melastatin 4 (Trpm4) channel. *J Biol Chem* 2013; **288**: 3655–3667.
- Gerzanich V, Woo SK, Vennekens R, Tsybalyuk O, Ivanova S, Ivanov A *et al*. De novo expression of Trpm4 initiates secondary hemorrhage in spinal cord injury. *Nat Med* 2009; **15**: 185–191.
- Simard JM, Woo SK, Norenberg MD, Tosun C, Chen Z, Ivanova S *et al*. Brief suppression of Abcc8 prevents autodestruction of spinal cord after trauma. *Sci Transl Med* 2010; **2**: 28ra29.
- Simard JM, Tsybalyuk O, Keledjian K, Ivanov A, Ivanova S, Gerzanich V. Comparative effects of glibenclamide and riluzole in a rat model of severe cervical spinal cord injury. *Exp Neurol* 2012; **233**: 566–574.
- Simard JM, Popovich PG, Tsybalyuk O, Gerzanich V. Spinal cord injury with unilateral versus bilateral primary hemorrhage—effects of glibenclamide. *Exp Neurol* 2012; **233**: 829–835.
- Soblosky JS, Song JH, Dinh DH. Graded unilateral cervical spinal cord injury in the rat: evaluation of forelimb recovery and histological effects. *Behav Brain Res* 2001; **119**: 1–13.
- Simard JM, Chen M, Tarasov KV, Bhatta S, Ivanova S, Melnitchenko L *et al*. Newly expressed SUR1-regulated NC(Ca-ATP) channel mediates cerebral edema after ischemic stroke. *Nat Med* 2006; **12**: 433–440.
- Simard JM, Yurovsky V, Tsybalyuk N, Melnichenko L, Ivanova S, Gerzanich V. Protective effect of delayed treatment with low-dose glibenclamide in three models of ischemic stroke. *Stroke* 2009; **40**: 604–609.
- Simard JM, Woo SK, Tsybalyuk N, Voloshyn O, Yurovsky V, Ivanova S *et al*. Glibenclamide–10-h treatment window in a clinically relevant model of stroke. *Transl Stroke Res* 2012; **3**: 286–295.
- Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995; **12**: 1–21.
- Bilgen M, Abbe R, Liu SJ, Narayana PA. Spatial and temporal evolution of hemorrhage in the hyperacute phase of experimental spinal cord injury: in vivo magnetic resonance imaging. *Magn Reson Med* 2000; **43**: 594–600.
- Hackney DB, Ford JC, Markowitz RS, Hand CM, Joseph PM, Black P. Experimental spinal cord injury: MR correlation to intensity of injury. *J Comput Assist Tomogr* 1994; **18**: 357–362.
- Ohta K, Fujimura Y, Nakamura M, Watanabe M, Yato Y. Experimental study on MRI evaluation of the course of cervical spinal cord injury. *Spinal Cord* 1999; **37**: 580–584.
- Weirich SD, Cotler HB, Narayana PA, Hazle JD, Jackson EF, Coupe KJ *et al*. Histopathologic correlation of magnetic resonance imaging signal patterns in a spinal cord injury model. *Spine* 1990; **15**: 630–638.
- Balentine JD. Pathology of experimental spinal cord trauma. I. The necrotic lesion as a function of vascular injury. *Lab Invest* 1978; **39**: 236–253.
- Aarabi B, Simard JM, Kufera JA, Alexander M, Zacherl KM, Mirvis SE *et al*. Intramedullary lesion expansion on magnetic resonance imaging in patients with motor complete cervical spinal cord injury. *J Neurosurg Spine* 2012; **17**: 243–250.
- Popovich PG, Lemeshow S, Gensel JC, Tovar CA. Independent evaluation of the effects of glibenclamide on reducing progressive hemorrhagic necrosis after cervical spinal cord injury. *Exp Neurol* 2012; **233**: 615–622.
- Mishra MK, Ray D, Barik BB. Microcapsules and transdermal patch: a comparative approach for improved delivery of antidiabetic drug. *AAPS Pharm Sci Tech* 2009; **10**: 928–934.
- Tosun C, Koltz MT, Kurland DB, Ijaz H, Gurakar M, Schwartzbauer G *et al*. The Protective Effect of Glibenclamide in a Model of Hemorrhagic Encephalopathy of Prematurity. *Brain Sci* 2013; **3**: 215–238.
- Tator CH, Hashimoto R, Raich A, Norvell D, Fehlings MG, Harrop JS *et al*. Translational potential of preclinical trials of neuroprotection through pharmacotherapy for spinal cord injury. *J Neurosurg Spine* 2012; **17** (Suppl I), 157–229.
- Wahl F, Stutzmann JM. Neuroprotective effects of riluzole in neurotrauma models: a review. *Acta Neurochir Suppl* 1999; **73**: 103–110.
- Wu Y, Satkunendrarajah K, Teng Y, Chow DS, Buttigieg J, Fehlings MG. Delayed post-injury administration of riluzole is neuroprotective in a preclinical rodent model of cervical spinal cord injury. *J Neurotrauma* 2013; **30**: 441–452.
- Grossman RG, Fehlings M, Frankowski R, Burau KD, Chow D, Tator C *et al*. A prospective multicenter phase 1 matched comparison group trial of safety, pharmacokinetics, and preliminary efficacy of riluzole in patients with traumatic spinal cord injury. *J Neurotrauma* (e-pub ahead of print 16 July 2013).



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1 Wyndaele M, Wyndaele JJ. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord* 2006; **44**: 523–529.